YEAST $tRNA^{Leu}$ (ANTICODON U–A–G) TRANSLATES ALL SIX LEUCINE CODONS IN EXTRACTS FROM INTERFERON TREATED CELLS

Jean WEISSENBACH and Guy DIRHEIMER

Institut de Biologie Moléculaire et Cellulaire du CNRS and *Faculté de Pharmacie, Université Louis Pasteur, 15 Rue René Descartes, 67084 Strasbourg Cedex*

and

Rebeca FALCOFF, Josiane SANCEAU and Ernesto FALCOFF

Fondation Curie, Institut du Radium, 26 Rue d'Ulm, 75005 Paris, France

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1. Introduction

Preincubation of extracts from interferon treated cells results in an impairment of translation of exogenous natural and synthetic messenger RNAs [1–5]. This inhibition can be reversed by the addition of eukaryotic tRNAS [5–7] and the restoring tRNA species can be charged with leucine [5,6,8]. On the other hand it has been shown that in these extracts endogenous leucine tRNAs are inactivated during incubation [9]. In this paper we show a selective degradation of tRNA in incubated extracts, and an interferon-dependent inactivation of $tRNA^{Leu}$ and report that a single purified $tRNA^{Leu}$ species from yeast is able to restore the translation of all the synthetic and natural mRNAs tested. Since leucine is coded by 6 codons, these results cannot be explained by the wobble hypothesis [10] alone. We have characterized the restoring $tRNA^{Leu}$; it is identical to the $tRNA^{Leu}$ sequenced by Randerath et al. [11]. We show that although its anticodon is U–A–G it is able to translate the 6 codons of leucine.

2. Materials and methods

2.1. *Materials*

Polyribonucleotides were purchased from Miles. The base ratio of the random copolymers was 1 : 1. Brewer's yeast and calf liver tRNAs were from Boehringer (Mannheim).

2.2. *Purification of $tRNA^{Leu}$ (anticodon U–A–G) [11] and sequencing techniques*

$tRNA^{Leu}_{UAG}$ was obtained by a three step purification. The first step was a counter-current distribution of brewer's yeast tRNA following the procedure of Holley et al. [12] as described by Dirheimer and Ebel [13]. Fractions corresponding to $tRNA_3^{Arg}$ [14] were then chromatographed on a Sepharose 4B column in conditions slightly different from Holmes et al. [15] (fig.1A). The major leucine accepting peak was finally submitted to RPC 5 [16] column chromatography (fig.1B). The T_1 RNAase digest of $tRNA^{Leu}_{UAG}$ has been analyzed as previously described [17]. $tRNA_3^{Leu}$ (anticodon C–A–A) [18] was a gift from Dr G. Pixa from our laboratory in Strasbourg.

2.3. *Cell-free extracts and protein synthesizing conditions*

Lysates from mouse cells were prepared as already described [19] with some modifications and will be referred to as Cont S-10 when derived from untreated cells and Int S-10 when derived from cells pretreated with homologous interferon. The modifications introduced are the following: 20 mM Hepes, pH 7.4 (instead of Tris–HCl buffer), 3.35 mM $Mg(AcO)_2$ and 0.5 mM spermidine. Protein syntheses were

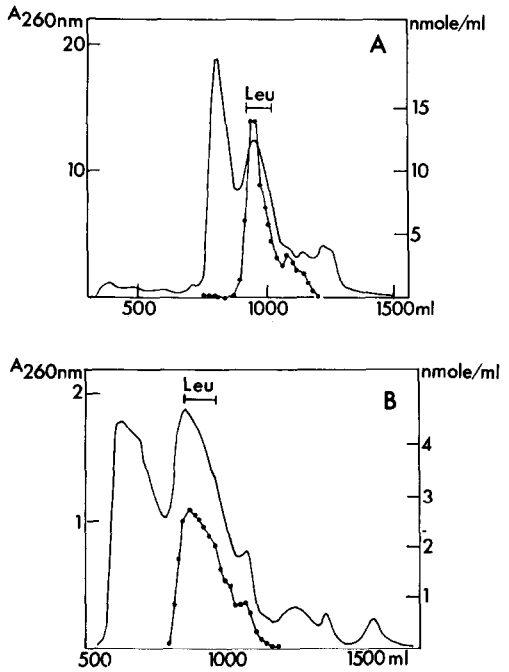

Fig.1. Purification of $tRNA^{Leu}_{UAG}$ with 200 mg enriched counter-current distribution fractions. (A) Sepharose 4B column chromatography using a reverse ammonium sulphate gradient from 2–1 M at 20°C in NaAcO 10 mM, pH 4.5, $MgCl_2$ 10 mM, β-mercaptoethanol 6 mM, EDTA 1 mM. Column size: 50 × 2.5 cm. Total gradient vol.: 2000 ml. Flow rate: 60 ml/h. (B) RPC 5 column chromatography of 35 mg 'Leu' fraction coming from the Sepharose 4B chromatography (A) using a sodium chloride gradient from 0.35–0.75 M in NaAcO 20 mM, pH 4.5, $MgCl_2$ 10 mM, EDTA 1 mM. Column size: 220 × 1.5 cm. Total gradient vol.: 2000 ml. Flow rate: 145 ml/h.

carried out in 25 μl under the following conditions:

(i) For 1 μg Mengo RNA: 2 mM Mg^{2+} and 100 mM K^+.
(ii) For 20 μg poly(U,C): 4 mM Mg^{2+}, 110 mM K^+.
(iii) For 6 μg poly(U): 4 mM Mg^{2+}, 100 mM K^+.
(iv) For 10 μg poly(U,G) or poly(U,A): 4 mM Mg^{2+}, 110 mM K^+.

Other components were as in ref. [19].

2.4. *Binding experiments*

2.4.1. *Escherichia coli* ribosomes

tRNA–ribosome binding experiments were carried out in 50 μl adjusted to 50 mM Tris–AcOH (pH 7.2), 10 mM $Mg(AcO)_2$, 50 mM NH_4Cl with 1 A_{260} unit of *E. coli* 70 S 'tight couple' ribosomes prepared according to Noll et al. [20], 20 pmol [^{14}C]leucyl-tRNA (spec. act. 250 cpm/pmol) and 20 pmol phosphate residues of polynucleotide. Ribosomes were a generous gift from Dr Vormbrock (Darmstadt). Assays were incubated for 15 min at 25°C, then diluted with 3 ml 50 mM Tris–AcOH, pH 7.2, 10 mM $Mg(AcO)_2$, 50 mM NH_4Cl ice-cold buffer, filtered on Sartorius SM 11406 membrane filters and washed with the same buffer. Filters were dried and counted in toluene omnifluor.

2.4.2. Yeast ribosomes

Yeast ribosomes were a kind gift from Dr Robertson (Munich). Binding experiments were done as described by Robertson et al. [21], using tRNA and polynucleotide concentrations specified above in 50 μl media containing 1.3 A_{260} unit of ribosomes.

2.4.3. L cell ribosomes

Binding was done using Zilberstein's conditions [8] except 1 A_{260} unit of ribosomes was used, the tRNA and polynucleotide concentrations are given above.

2.5. *tRNA extraction from cell extracts*

tRNA from L cell extracts was prepared according to Aviv et al. [22].

3. Results

3.1. *Selective degradation of tRNAs during preincubation*

It has been reported [9] that during the preincubation of cell extracts, some tRNA species, especially those corresponding to leucine, lysine and serine, were inactivated, i.e. they could no longer be charged with their corresponding amino acids. This inactivation was faster and more complete in interferon treated cells than in the controls.

We have extracted the tRNAs from Cont S-10 and Int S-10 before and after 90 min preincubation. The results (table 1) show that the yield of tRNA from incubated cell extracts diminishes by about 50% regardless of the pretreatment of the cells. This degradation is selective because it does not affect all

Table 1
Leucine and arginine acceptance by tRNAs isolated from interferon-treated and control cell lysates before and after incubation[a]

Source of tRNA	Yield of total tRNA[b] (A_{260} units)	Chargeable $tRNA^{Leu}$ [c] (pmol)	Chargeable $tRNA^{Arg}$ [c] (pmol)	$tRNA^{Leu}$ (%)	$tRNA^{Arg}$ (%)
Cont S-10 not preincubated	2.26	137	152	3.8	4.2
Int S-10 not preincubated	2.09	120	144	3.6	4.5
Cont S-10 preincubated	1.26	40	196	2.0	9.7
Int S-10 preincubated	1.19	10	186	0.5	9.8

[a]90 min
[b]Extracted from 1 ml lysate
[c]Measured by aminoacylation of extracted total tRNA

the tRNAs, for instance the charging capacity of $tRNA^{Arg}$ is enhanced. This enhancement remains to be explained.

On the other hand preincubation leads to a 3.5-fold loss of leucine accepting capacity in the Cont S-10 and to a 12-fold loss in the Int S-10.

This diminished amount of active $tRNA^{Leu}$ may account for the need for added $tRNA^{Leu}$ to overcome the impairment of translation in incubated Int S-10. For instance, poly(U,G)-directed polypeptide synthesis which is inhibited in preincubated Int S-10 is restored by the addition of yeast $tRNA_3^{Leu}$ (anticodon C–A–A) while the translation of Mengo RNA is not restored (results not shown). On the contrary we have shown that another yeast $tRNA^{Leu}$ fraction releases this inhibition [5]. We have now characterized this tRNA.

3.2. *Isolation and identification of the restoring $tRNA^{Leu}$*

The $tRNA^{Leu}$ was isolated as indicated under Materials and methods (fig.1), identified by its T_1 RNAase digest products which were separated by DEAE-cellulose paper electrophoresis, eluted, hydrolysed to nucleosides which were characterized by two-dimensional thin-layer chromatography and quantitated by ultraviolet spectra [17,23]. All these digest products were identical to those expected after T_1 RNAase digestion of $tRNA^{Leu}$ (anticocon U–A–G) sequenced by Randerath et al. [11]. This tRNA had been named $tRNA^{Leu}_{CUA}$ by these authors because CUA is the complementary codon to the anticodon UAG. However as will be shown below, this is not the only codon recognized by the tRNA. We prefer therefore to designate it as $tRNA^{Leu}_{UAG}$.

3.3. *Restoration by $tRNA^{Leu}_{UAG}$ of the translation of different mRNAs*

We have checked the restoring capacity of $tRNA^{Leu}_{UAG}$ with different mRNAs (table 2). With Mengo RNA as messenger this tRNA restores the translation in incubated Int S-10 to the levels of the control. When total tRNA is added the activity is higher than with $tRNA^{Leu}_{UAG}$ probably because it overcomes the degradation of several endogenous tRNAs which occurs during the preincubation.

In Int S-10 supplemented with $tRNA^{Leu}_{UAG}$ or total calf liver tRNA the polypeptides synthesized during the translation of Mengo RNA are the same as those synthesized in Cont S-10, as judged by polyacrylamide gel electrophoresis (results not shown).

With poly(U,C), poly(U,A) and poly(U,G) as messenger RNAs which contain the leucine codons: C–U–U, C–U–C, U–U–A and U–U–G, the polypeptide synthesis is inhibited in Int S-10 and can be completely restored by the $tRNA^{Leu}_{UAG}$. This effect is quite surprising since $tRNA^{Leu}_{UAG}$ should only recognize C–U–A and C–U–G according to the wobble hypothesis. This result will be discussed further on.

Table 2
tRNA-dependent translation of different messenger RNAs in preincubated extracts from interferon-treated cells

mRNA	Added tRNA (μg/ml)		[^{14}C]Leucine incorporated into TCA-insoluble polypeptides in 90 min		
	$tRNA^{Leu}_{UAG}$	Total tRNA[a]	Incubated Cont S-10 (cpm)[b]	Incubated Int S-10 (cpm)[b]	% Respective control
Mengo RNA	–	–	6696	1895	(28)
	2	–	6201	3114	(50)
	4	–	5914	4369	(70)
	20	–	5692	6102	(107)
		40	10 434	10 288	(99)
Poly(U,C)	–	–	3679	1089	(26)
	2	–	3650	3471	(94)
	4	–	4035	3863	(97)
		40	4897	5055	(104)
Poly(U,A)	–	–	138	11	(8)
	8	–	199	186	(93)
		40	187	222	(119)
Poly(U,G)	–	–	671	130	(19)
	8	–	885	700	(79)
	40	–	1472	1423	(97)
	–	40	1478	1656	(112)
Poly(U)[c]	–	–	4114	150	(4)
	8	–	4172	500	(12)
	40	–	5376	1390	(18)
	80	–	5829	2096	(36)
		40	3847	2781	(72)

[a]Calf liver
[b]Reaction mixture, 20 μl, background substracted
[c][^{14}C]Leucine is the only amino acid present in the reaction mixture

The poly(U)-directed polyleucine synthesis has to be ascribed to misreading by leucine tRNAs. This misreading is also impaired in incubated Int S-10. It can be only partially restored by the $tRNA^{Leu}_{UAG}$.

3.4. *Binding properties of* $tRNA^{Leu}_{UAG}$

Binding experiments were carried out with ribosomes from *E. coli*, yeast and L cells in the presence of different tri- and polynucleotides. As expected from its anticodon, $tRNA^{Leu}_{UAG}$ binds to ribosomes from L cells in presence of C–U–A and C–U–G (results not shown). Good binding is achieved with poly(U,C) whatever the ribosomal source. This is in accord with the results of poly(U,C) translation, suggesting that the anticodon U–A–G of $tRNA^{Leu}_{UAG}$ pairs with C–U–U and or C–U–C and implying U·C and/or U·U pairing in the wobble position. Since poly(U,A) and poly(U,G) are translated we expected binding with these polynucleotides, but as shown in table 3, this is not obtained with either *E. coli* or L cell ribosomes. However yeast ribosomes show a very low binding. A low binding is observed with poly(U) which could explain its misreading as shown in table 2.

4. Discussion

According to the wobble hypothesis $tRNA^{Leu}_{UAG}$ should be specific for C–U–A and C–U–G codons.

Table 3
Binding properties of $tRNA^{Leu}_{UAG}$ to synthetic polynucleotides (cpm)

Polyribonucleotides	None	Poly(U,C)	Poly(U,A)	Poly(U,G)	Poly(U)
E. coli ribosomes	231	1180	238	160	350
Yeast ribosomes	96	1626	167	264	387
L cell ribosomes	402	2072	433	403	432

It is striking to notice that this tRNA is also able to translate C–U–C and/or C–U–U codons. Binding experiments with poly(U,C) show that the codon–anticodon association is still relatively strong. These results suggest the existence of U·C and/or U·U base pairs between the wobbling base of the anticodon and the third base of the codon. This situation has not been completely ruled out by Crick [10]. An indirect indication of a C·C pairing in the wobble position has also been obtained recently for leucine tRNA of *E. coli* [24]. Another pairing, not predicted by the wobble hypothesis and concerning unmodified bases, has been reported recently for *E. coli* valyl-tRNA (anticodon G–A–C) which was able to translate MS2 RNA the sequence of which bears all four valine codons [25]. In this case G in the wobble position of the anticodon should also pair with A and G in the third place of the codon. The other explanation, favored by these authors, would be that, under their in vitro translation conditions, the genetic code for valine is built of the first two letters only.

Under our conditions the translation of poly(U,C) involves either U·U and/or U·C pairing in the wobble position or a code of two letters, i.e., C–U for leucine.

Although no binding was obtained between $tRNA^{Leu}_{UAG}$ and poly(U,A) or poly(U,G) a restoration of their translation was obtained. This result is very unexpected and leads us to assume that pairing ambiguities (existence of G·U pairs) take place between the first letter of a codon and the third letter of the anticodon. Such ambiguities have been predicted by Ninio [26] in his 'missing triplet hypothesis'. However the ambiguities described by Ninio involve the presence of a neighbouring G·C pair, which is not the case in this system. Moreover, in addition to the classical initiator codon A–U–G, G–U–G and U–U–G can also be used as reinitiator codons in vivo [27] and correspond to $tRNA^{Met}_i$ (anticodon C–A–U). This also implicates G·U and even U·U pairs between the first position of the codon and the 3′-position of the anticodon.

Concerning the translation of poly(U) our results suggest that less misreading occurs with $tRNA^{Leu}_{UAG}$ than with the endogenous leucine tRNA population since the reversion is only partial.

Finally our results show that studies of the binding of tRNAs do not always give a conclusive answer as to their utilisation in protein synthesis.

All these coding properties of $tRNA^{Leu}_{UAG}$ could be shown in an in vitro protein synthesizing system from interferon treated cells. The preincubation of this extract leads to inactivation of nearly all the endogenous leucine tRNAs. This system is therefore dependant on added $tRNA^{Leu}$. The particular property of reading all six leucine codons shown by $tRNA^{Leu}_{UAG}$, which is a major species of yeast $tRNA^{Leu}$, may explain why a single tRNA is able to restore the translation activity in preincubated Int S-10 of a variety of messenger RNAs particularly of Mengo RNA.

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